0031-3998/11/6902-0123
PEDIATRIC RESEARCH

Vol. 69, No. 2, 2011
Printed in U.S.A.

Enteral Feeding *In Utero* Induces Marked Intestinal Structural and Functional Proteome Changes in Pig Fetuses

PINGPING JIANG, JENNIFER MAN-FAN WAN, WAI-HUNG SIT, CHEUK-LUN LEE, METTE SCHMIDT, AND PER T. SANGILD

School of Biological Sciences [P.J., J.M.-F.W., W.-H.S., C.-L.L.], The University of Hong Kong, Hong Kong (SAR), People's Republic of China; Department of Human Nutrition [M.S., P.T.S.], University of Copenhagen, Frederiksberg C, DK-1958, Denmark

ABSTRACT: Intestinal adaptation from parenteral to enteral nutrition is crucial for survival and growth of newborns. Rapid feeding-induced gut maturation occurs immediately after birth in both preterm and term neonates, but it remains unclear whether the responses depend on factors related to birth transition (*e.g.* bacterial colonization, endocrine, and metabolic changes). We hypothesized that enteral feeding matures the immature intestine, even in fetuses before birth. Hence, control pig fetuses were compared with fetuses fed with milk formula for 24 h *in utero*. Gel-based proteomics showed that feeding-induced changes in 38 proteins, along with marked increases in intestinal mass and changes in activities of brush border enzymes. Physiological functions of the identified proteins were related to enterocyte apoptosis (*e.g.* caspase 1) and nutrient metabolism (*e.g.* citric acid cycle proteins). Many of the differentiated proteins were similar to those identified previously in preterm pigs fed with the same formula after birth, except that effects on proteins related to inflammatory lesions (*e.g.* heat shock proteins) were absent. Our results show that enteral feeding, independently of the birth transition, induces marked gut maturation and proteome change in the immature intestine. Hence, immediate postnatal feeding-induced gut changes are largely independent of factors related to the birth transition. **(*Pediatr Res* 69: 123–128, 2011)**

Enteral feeding markedly affects the intestine in preterm neonates, and the changes may not only be important for the adaptation to enteral nutrient intake but may also provoke severe intestinal complications, such as necrotizing enterocolitis (NEC) (1). In our previous proteomic studies on the preterm pig intestines, aspects such as antioxidation and detoxification, nutrient metabolism, and some signal transduction pathways were disturbed after newborn pigs were fed with formula that led to variable degrees of NEC lesions (2,3). These feeding associated proteome changes after just 1 d of feeding may have been induced by feeding alone or by the combined effects of feeding and microbial colonization, physiological changes at birth, and the progression of NEC inflammatory lesions. However, it seems that it is not factors such as mode of birth (4), *in utero* growth restriction (5), or bacterial flora (6) but rather the amount and type of feeding that determine the intestinal response just after birth (7). Feeding *per se* may be the main drive for maturation of neonatal intestines. Hence, we hypothesized that feeding alone would stimulate the growth and affect the proteome of the immature fetal intestine, independently of the later birth transition, bacterial colonization, and feeding-induced NEC lesions.

To prove our hypothesis, we used short-term oral feeding of fetal pigs *in utero*, having the same GA and feeding regimen (diet, amount, and frequency) as in the previous studies on postnatal preterm pigs (1–3,5,6,8). Gel-based expression proteomics was used to assess the expression of hundreds of different intestinal proteins simultaneously. We could thereby indicate that molecular pathways in the immature intestine were most clearly affected by feeding alone, in addition to some standard parameters of intestinal mucosal structure and digestive enzyme function.

MATERIALS AND METHODS

Animals. Three pregnant sows (Large White × Landrace, 105 d gestation, term = 116 d) were anesthetized (5–10 mg/kg i.v. thiopental sodium; Abbott Laboratories, North Chicago, IL). After exposure of the uterus, two fetuses per sow were chosen randomly for surgery and later formula feeding. A small incision (5 cm) was made in a relatively nonvascular area of the uterus. A silastic catheter (vinyl tube, inner diameter of 0.86 mm, outer diameter of 1.52 mm; Dural Plastics and Engineering, Auburn, Australia) was passed down the esophagus of fetuses with the tip reaching the stomach. Antibiotics (2 mg/kg enrofloxacin, Baytril; Bayer, Leverkusen, Germany) were administered to the amniotic cavity of each fetus. After the surgery, all of the sows were given antibiotics (20 mL/d i.m. streptocillin; Boehringer Ingelheim, Copenhagen, Denmark) and progesterone (50 mg/d i.m.; University Pharmacy, University of Copenhagen, Denmark). After return of the sow to its crate, the two catheterized fetuses were fed with 15 mL enteral feeding formula every 3 h for 24 h (EF group, $n = 6$). On the basis of our observations in several previous studies on intestinal structure and function after feeding fetal pigs *in utero* (9–11), we did not include a separate group fed with saline. The effects of fetal surgery alone on intestinal physiology in similar aged pig fetuses are minimal, especially when the time from fetal surgery to later cesarean section is short (only 1 d in this study). All procedures were approved by the Danish National Committee on Animal Experimentation.

The formula was designed to match the macronutrient composition of sow's milk during lactation and identical to that used previously to feed preterm pigs with a similar GA at delivery (2,3,6). Each liter of the formula contained 80 g Peptide 2–0, 70 g Maxipro, and 75 mL Liquigen-MCT (SHS, Liverpool, United Kingdom; all products were donated by SHS). Major ingredients in the formula were hydrolyzed dried maize syrup (maltodextrins), bovine whey protein, hydrolyzed meat and soya protein, and oils from maize and coconut (medium-chain triglycerides). Macronutrient composition was (per liter formula) as follows: 64 g protein, 47 g total carbohydrate, 3 g lactose, and 61 g fat. Before feeding, the formula was sterilized by γ-irradiation (2 × 5.0 kGy; LR Plast, Glostrup, Denmark).

Tissue collection. After feeding, the fed fetuses (EF group, $n = 6$) were delivered by cesarean section (6), together with some random unfed control

Received July 1, 2010; accepted September 15, 2010.

Correspondence: Per T. Sangild, D.M.Sc., D.V.Sc., Ph.D., Department of Human Nutrition, Faculty of Life Sciences, University of Copenhagen, 30 Rolighedsvej, Frederiksberg C, DK-1958, Denmark; e-mail: psa@life.ku.dk

Supported by the Danish Research Councils and The University of Hong Kong.

Abbreviations: 2-DE, two-dimensional gel electrophoresis; **Ap,** amino peptidase; **DPPIV,** dipeptidyl peptidase IV; **HIF-1α,** hypoxia-inducible factor-1α; **HSP,** heat shock protein; **NEC,** necrotizing enterocolitis

fetuses (CTR group, $n = 6$). The small intestine from the pyloric sphincter to the ileocecal junction was rapidly excised by cutting along the mesenteric border. The small intestine was carefully emptied of its contents and divided into three equally long segments, designated proximal, middle, and distal small intestine. The middle part (2–3 cm) of middle small intestine was snap frozen in liquid nitrogen and saved at −80°C for later proteomic studies or fixed in 4% paraformaldehyde for 48 h and then transferred to 70% ethanol to be stored at 4°C for later histological preparation. The proportion of mucosa was recorded by gently scraping off the mucosal layer of a 10-cm intestinal piece (proximal, middle, and distal) and determining its proportional dry weight of the intact intestine after drying both at 65°C.

Intestinal morphology and enzyme activities. The paraformaldehyde fixed samples were embedded in paraffin and sectioned at 3 μm before mounted on the slides for staining with hematoxylin and eosin. All slides were checked with a light microscope (Orthoplane, Leitz) and analyzed by NIH Image J software (National Institutes of Health, MD).

Frozen middle intestinal sections were extracted in 1.0% Triton X-100 (10 mL/g tissue) and homogenized (0°C, 20 s). The homogenates were assayed for activities of lactase, sucrase, maltase, dipeptidyl peptidase IV (DPPIV), amino peptidase A (ApA), and peptidase N (ApN) as described previously (1). For all enzymes, a hydrolytic rate of 1 μmol substrate per minute at 37°C was regarded as one unit of enzyme activity.

Protein extraction. Extraction of intestinal protein was performed as previously described (2,3). Briefly, the tissue samples were disrupted with a tissue teaser (BioSpec Products, Bartlesville, OK) in a cocktail buffer (1% Triton X-100, 25 mM HEPES, 150 mM NaCl, 1 mM EDTA disodium salt, and 1 mM DTT) with Protease Inhibitor Cocktail Set III (Bio-Rad, Hercules, CA). The protein extracts were obtained by centrifuging the homogenates at 15,800 × g for 30 min at 4°C. The superfluous salt in the extracts were removed by precipitation with trichloroacetic acid-acetone solution and acetone contained 20 mM DTT. The protein pellet was resuspended in a buffer containing 7 M urea, 2 M thiourea, 4% Chaps, 100 mM DTT, and 5% glycerol. The protein solution was stored at −80°C until further analysis of two-dimensional gel electrophoresis (2-DE). Protein concentration of the samples was determined by Bio-Rad Protein Assay (Bio-Rad).

2-DE. The protein sample (100 μg) was mixed with rehydration buffer (9.5 M urea, 2% Chaps, 0.28% DTT, and 0.5% IPG buffer pI 3–10) and applied onto one ReadyStrip IPG Strip (18 cm, pI 3–10 NL, Bio-Rad). The isoelectric focusing was carried out on an Ettan IPGphor III IEF System (GE Healthcare) after an active rehydration for 7 h, after the program, *i.e.* linearly running up to 500 V in 1 h, holding at 500 V for 3 h, after linearly increasing up to 10,000 V in 3 h, and holding at 10,000 V to reach a total of 90,000 Vh (V × h) (2,3). The focused gel strips were placed onto 1.0-mm-thick 12.5% PAGE gels, and SDS-PAGE was carried out in a PROTEAN xi II cell (Bio-Rad), after a two-step equilibration with a buffer (6 M urea, 30% glycerol, and 2% SDS in 50 mM pH 8.8 Tris-HCl) containing 1% DTT and then with 2.5% iodoacetamide successively.

Gel image analysis and protein identification. After electrophoresis, gels were stained with SYPRORuby Protein Stain (Bio-Rad) according to the manufacturer's guide. The stained gels were scanned with a Molecular Imager PharosFX Plus System (Bio-Rad) and analyzed by PDQuest 8.0 (Bio-Rad) according to the following procedures: background subtraction, spot detection, and spot matching. Matched spots were assigned with numbers automatically. The in-gel trypsin digestion of the manually cut-out gel spots was performed as described previously (3). The resulting peptide mixtures were applied on a 4800 MALDI TOF/TOF Analyzer (Applied Biosystem, Carlsbad, CA) for MALDI-TOF/TOF MS (matrix-assisted laser desorption ionization-time of flight/time of flight mass spectrometry). The identity searching was based on the MS spectrum obtained is a combination of peptide mass fingerprint and MS/MS search using in-house searching engine, Mascot, with searching taxonomy limited as *Mammalia* (mammals) against NCBInr database (ftp://ftp.ncbi.nih.gov/blast/ db/README). A protein match with a protein score >71 was considered significant.

Western blot analysis. Briefly, 25 μg protein extracted was resolved by electrophoresis. Anti-glutathione synthetase, anti-caspase 1, and anti-calreticulin (Abcam, Cambridge, United Kingdom) were used to detect the expression of glutathione synthetase, caspase 1, and calreticulin as three examples of proteins from the proteomics analysis that were markedly affected by feeding *in utero*. The protein bands were visualized, and the density of the protein bands was detected by Quantity One (Bio-Rad). The lack of monospecific antibodies for most porcine protein variants precluded a large- scale Western blot analysis of differentially regulated proteins.

Statistical analysis. All data are expressed as mean ± SEM. Two-tailed *t* test with Levene's test for equality of variances was used to compare the expression level of proteins by proteomics and Western blot analysis. SPSS 11.5 was used for all statistical analyses with p value <0.05 considered as significant.

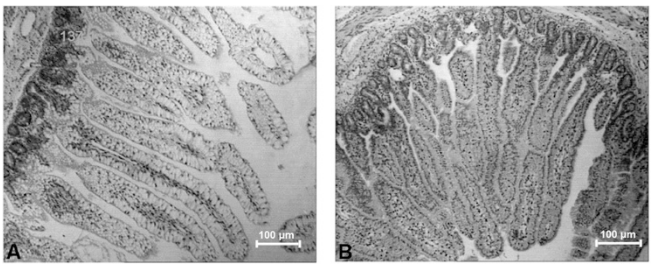

Figure 1. Representative histopathology of the intestine in CTR (*A*) and EF (*B*) pigs (magnification = ×200). Hematoxylin and eosin stained jejunum sections show normal intact villus structures of both groups of pigs with no signs of histopathology or mucosal damage. The EF group showed a trophic responses to feeding involving some villus swelling (marginal increase in villus width and length, see also Fig. 2) and empty vacuolated cells.

RESULTS

Intestinal structure and enzyme function. All pigs were well and alive when delivered by cesarean section 24 h after the start of enteral feeding. No pigs showed any macroscopic signs of NEC. Representative hematoxylin and eosin stained histological graphs of the intestine in EF and CTR groups are showed in Figure 1. The examinations showed that there were no significant differences in villus height and crypt depths between groups, whereas villus width showed a small increase in the EF pigs relative to the controls ($p < 0.05$). Furthermore, the EF piglets showed higher weights of stomach (+24%, $p < 0.05$), intestine (+55%, $p < 0.01$), and specifically the mucosa part (+75%, $p < 0.001$) than CTR piglets (Fig. 2*A*). The EF piglets also showed a tendency to a longer intestine (303 *versus* 240 cm/kg, $p = 0.07$) and a larger circumference (10.7 *versus* 8.3 mm, $p < 0.001$). The activities of ApA (+54%, $p < 0.001$) and maltase (+40%, $p < 0.05$) were found higher, whereas activities of lactase (−40%) and DPPIV (−60%) were found lower in EF piglets compared with CTR piglets (both $p < 0.001$, Fig. 2*B*).

Identified proteins with differential expression. Representative 2-DE gel graphs of the intestinal proteome from EF and CTR piglets are shown in Figure 3. The pI range of the gels was 3–10 and that of M_r 10–150 kD; ~500 spots were identified on each gel with the expression analyzing software. Among the detected spots, 38 proteins were successfully found with definite identity and the corresponding spots on the 2-DE gel graph were labeled with numbers as shown in Figure 3. Descriptive information of the identified proteins, including spot number showing the position of spots on gels, protein name, GenInfo identifier, protein score, searched and apparent pI and M_r, and expression change between EF and CTR groups, are showed in Table 1. Among the identified proteins, 18 proteins showed higher expression in EF piglets than that in CTR piglets with two of them (ferritin L subunit, spot 4010; cathepsin Y, spot 5208) having no detectable expression in the CTR piglets. The other 20 identified proteins showed lower expression in EF piglets, with one of these (caspase 1, spot 3208) showing expression below the detection limit in EF piglets. A detailed description of the most relevant groups of identified proteins is given in the Discussion.

Western blot. The relative abundance of three selected proteins were further tested by Western blot analysis (Fig. 4).

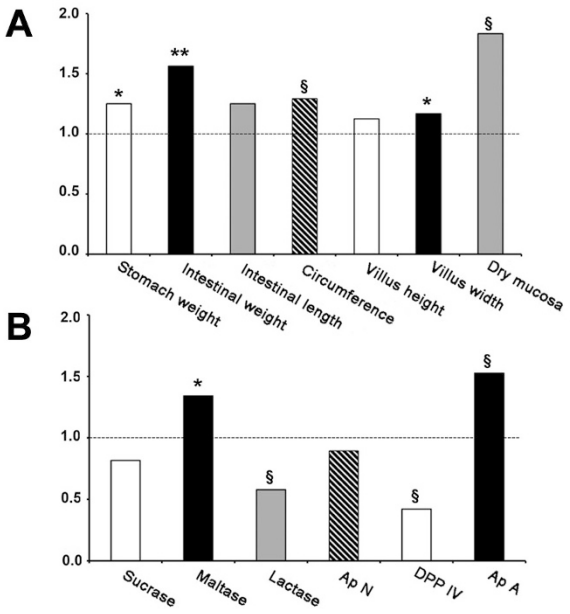

Figure 2. Gut morphology values (*A*) and intestinal brush border enzyme activities (*B*) in formula-fed (EF) pig fetuses relative to values in CTR fetuses (=1.0). Enteral feeding for 24 h significantly increased intestinal length (cm/kg body weight) and weight (g/kg body weight), circumference (mm), villus height and width (μm), and total amount of mucosa determined on a dry matter basis (g/kg body weight). Similarly, *in utero* feeding increased maltase and aminopeptidase A activities (U/g tissue), while decreased lactase and DPPIV activities (U/g tissue) (*$p < 0.05$; **$p < 0.01$; §$p < 0.001$).

Glutathione synthetase, caspase 1 p45, and calreticulin showed lower levels in EF pigs than in CTR pigs ($p < 0.05$).

DISCUSSION

The intestinal response to the first enteral feed plays a key role in neonatal adaptation and growth, for both preterm and term neonates. Soon after birth, enteral food tolerance is required to achieve adequate nutrient intakes for body growth, and a suboptimal adaptation may lead to maldigestion, poor mucosal protection from colonizing bacteria, and increased risk of NEC, particularly for preterm neonates. In this study, we documented that the immature intestine was highly responsive to enteral feeding, even before birth, and that marked growth and brush border enzyme maturational changes (in the range of $\pm$50%) occurred within just 24 h of fetal enteral feeding. These responses in fetuses were quantitatively and qualitatively similar to those reported previously after feeding preterm newborn pigs with the same quantity of the same formula (6). The changes do not arise from an effect of fetal surgery itself because such treatment have been documented in our earlier fetal studies not to induce any marked changes on fetal intestinal growth and function, relative to those by enteral feeding (1). However, our proteomic analyses indicate that the functional responses to formula feeding *in utero* differ at a number of points, from those reported previously for postnatal preterm pigs (2,3,12). The proteome changes in this study can be regarded as specific for the first feeding, independent of factors related to the birth transition (microbial colonization, metabolic and cardiovascular changes, thermoregulation, respiration, and a series of endocrine changes). Furthermore, the feeding related proteome changes *in utero* will not be connected with possible pathological proteome changes related to progression of NEC lesions, as others and we have shown that NEC in preterm neonates does not develop in the absence of bacterial stimuli (1,6).

Six proteins that showed an expression change similar to that in our previous studies on postnatal preterm pigs (2,3) are glyoxalase 1 (spot 0205), GDI 2 (spot 3509), carbamoylphosphate synthetase 1 (spot 6902), galactokinase 2 isoform 2 (spot 4507), aldehyde dehydrogenase, mitochondrial precursor (spot 3609), and chain A, medium-chain acyl-CoA dehydrogenase with 3-thiaoctanoyl-CoA (spot 6401). These proteins represent cellular functions at least partly related to detoxification, energy, and nutrient metabolism, apparently required to facilitate the tissue adaptation response to feeding. Glyoxalase 1 converts cytotoxic α-oxoaldehyde generated from the oxidation of carbohydrates and glycolysis into noncytotoxic D-lactate (13,14). Galactokinase 2 involves in the

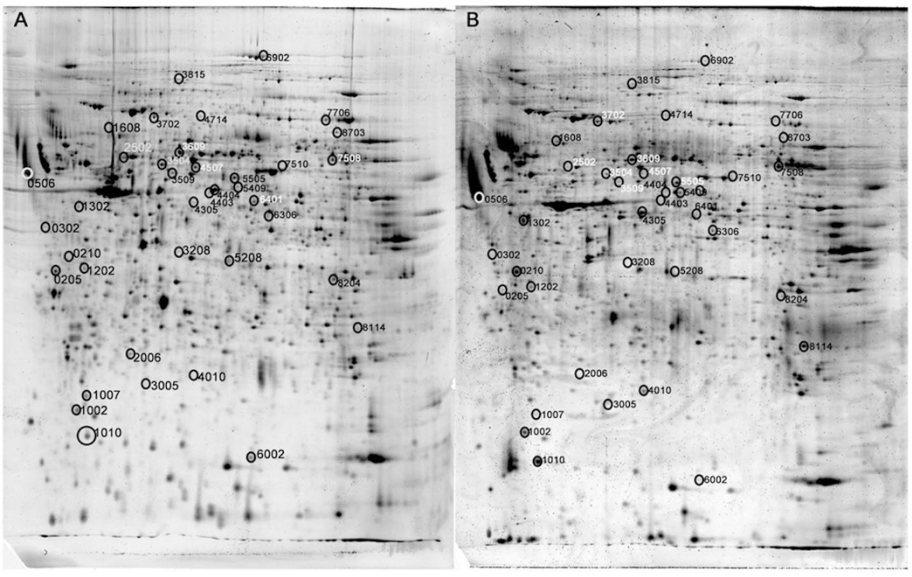

Figure 3. 2-DE gels of intestinal proteome map of CTR (*A*) and EF (*B*) piglets. Spot number was assigned by analysis software and correlated with the ones presented in Table 1.

Table 1. *Identified proteins with differential expression between EF and CTR pigs*

Spot no.*	Protein name	GenInfo identifier†	Protein score	pI (S/A)‡	M_r (kD) (S/A)	p§	Expression change EF/CTR
0205	Glyoxalase domain-containing protein 4	gi 81916755	118	5.3/4.5	33.6/32	0.0001	−5.0
0210	Actin, gamma 2 propeptide	gi 4501889	301	5.3/4.8	42.2/34	0.001	+3.7
0302	Alpha-2-HS-glycoprotein precursor	gi 231467	179	5.5/3.9	39.9/38	0.03	+3.8
0506	Calreticulin	gi 126723562	141	4.3/4.0	48.4/51	0.01	−2.0
1002	Elongation factor 1-gamma	gi 55977740	149	6.2/4.7	49.9/<20	0.003	+1.8
1007	PNAS-140	gi 12751117	129	4.6/4.9	17.2/<20	0.01	−2.7
1010	Eukaryotic initiation factor 5A isoform I variant A (eIF5A)	gi 33383425	68	6.5/4.9	20.4/<20	0.01	+1.9
1202	Keratin 9	gi 453155	89	5.1/4.9	62.2/32	0.001	+2.5
1302	Keratin 19	gi 90111766	88	5.0/4.7	44.1/42	0.01	+4.3
1608	Alpha-1-antitrypsin precursor	gi 47523844	286	5.5/5.3	47.4/65	0.06	−2.9
2006	Heme-binding protein	gi 58332866	148	5.7/5.4	21.2/24	0.06	−2.7
2502	CNDP dipeptidase 2	gi 31981273	84	5.4/5.3	53.2/55	0.004	−2.8
3005	Chain, ferritin (H-chain) mutant	gi 229918	136	5.2/5.4	21.4/24	0.002	+6.0
3208	Caspase 1	gi 47523412	113	6.1/5.8	45.5/35	N/A	−
3504	Glutathione synthetase	gi 4504169	182	5.7/5.8	52.5/52	0.004	−2.0
3509	GDP dissociation inhibitor 2	gi 48675953	112	6.3/5.9	50.7/49	0.006	−2.1
3609	Aldehyde dehydrogenase, mitochondrial precursor	gi 118502	310	7.6/6.0	57.1/56	0.001	−2.1
3702	Chain A, structure of complex with the hsc70 ATPase domain	gi 13399491	450	6.2/5.6	44.5/71	0.007	−3.8
3815	Keratin 10	gi 40354192	105	5.1/5.8	59.0/100	0.001	−3.0
4010	Ferritin L subunit	gi 10304378	78	5.8/5.9	18.3/21	N/A	+
4305	Alpha 2 actin	gi 4501883	332	5.2/5.9	42.4/43	0.02	+5.8
4403	Succinate-CoA ligase, ADP-forming, beta subunit	gi 55957259	87	6.6/6.0	48.4/45	0.0001	+2.8
4404	S-adenosylhomocysteine hydrolase	gi 58801555	67	5.9/6.0	48.2/46	0.0002	−3.6
4507	Galactokinase 2 isoform 2	gi 48527957	108	6.0/6.0	50.1/51	0.001	−2.5
4714	PRO2619	gi 11493459	166	6.0/5.9	58.5/72	0.007	+4.7
5208	Cathepsin Y	gi 34328540	127	6.7/6.0	34.8/34	N/A	+
5409	Pyruvate dehydrogenase: subunit alpha	gi 448580	155	6.8/6.2	41.0/47	0.01	+2.6
5505	GDP dissociation inhibitor 2	gi 48675953	385	6.3/6.1	50.7/49	0.04	+2.1
6002	Peroxiredoxin 5	gi 47523086	322	5.7/6.3	17.5/<20	0.01	−2.2
6306	Leukocyte elastase inhibitor	gi 417185	108	5.9/6.3	42.0/40	0.0001	+2.5
6401	Chain A, medium-chain acyl-CoA dehydrogenase with 3-thiaoctanoyl-CoA	gi 40889734	349	6.5/6.3	43.8/44	0.01	−2.0
6902	Carbamoyl-phosphate synthetase 1, mitochondrial	gi 8393186	170	6.3/6.4	165.7/150	0.01	−2.1
7508	Chain A, structure of glutamate dehydrogenase-apo form	gi 20151189	249	6.7/7.5	56.3/55	0.06	+2.1
7510	Similar to septin-11	gi 109499524	79	6.4/6.6	49.2/53	0.005	−2.6
7706	Phosphoenolpyruvate carboxykinase 2	gi 12655193	162	7.6/7.2	71.4/74	0.05	−2.9
8114	HNRPH1	gi 48145673	272	5.8/8.4	49.4/26	0.01	+1.8
8204	GNB2L1	gi 5174447	443	7.6/7.8	35.5/32	0.001	+1.9
8703	Bifunctional purine biosynthesis protein PURH	gi 20127454	67	6.3/7.9	65.1/67	0.003	−2.0

* Spot consistent with the ones presented in Figure 3.

† Sequence identification number by GenBank.

‡ Searched pI or M_r generated by MS platform and apparent pI or M_r calculated from gel graph aided by pI distribution curve provide by Bio-Rad.

§Probability that the mean of EF differs significantly from that in CTR ($p < 0.05$).

conversion of galactose to glucose (15), whereas aldehyde dehydrogenase is related to oxidation of aldehydes to carboxylic acids (16). Chain A, medium-chain acyl-CoA dehydrogenase with 3-thiaoctanoyl-CoA (spot 6401) catalyzes the mitochondrial fatty acid beta-oxidation (17), whereas pyruvate dehydrogenase α subunit (spot 5409), succinate-CoA ligase, ADP-forming, β unit (spot 4403), and chain A, structure of glutamate dehydrogenase-Apo form (spot 7508) are all enzymes involved in the citric acid cycle (18). Regulation at all these steps of energy metabolism may be required to facilitate the rapid tissue protein synthesis, as reflected also in increased elongation factor expression [eIF5A (eukaryotic translation initiation factor 5A, spot 1010), elongation factor 1-γ (spot 1002)], and the associated decrease in tissue proteolysis re-

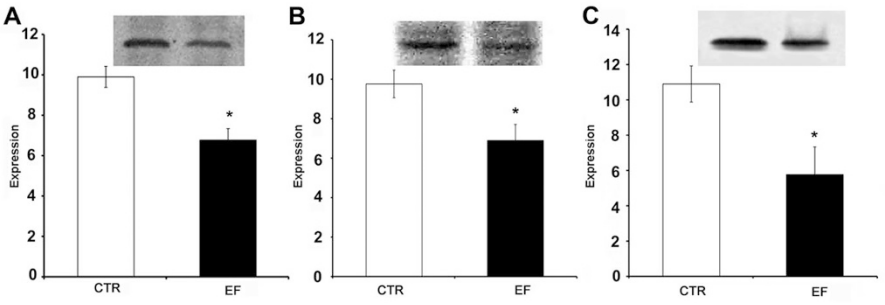

Figure 4. Western blot analysis of caspase 1 (*A*), glutathione synthetase (*B*), and calreticulin (*C*). Abundances of protein bands are presented as mean ± SEM $*p < 0.05$ in EF *vs* CTR.

flected by reduced cathepsin Y (spot 5208) and CNDP dipeptidase 2 (spot 2502) expression. Even before the birth transition, the immature intestine is capable of activating all these metabolic pathways to facilitate growth and functional adaptation. This observed adaptation could in part be specific to the specific formula composition used, *i.e.* the change in medium-chain acyl-CoA dehydrogenase could reflect a high level of vegetable medium-chain triglycerides in our formula, relative a diet such as mother's milk.

In postnatal preterm pigs, formula feeding rapidly increased apoptosis in the intestine and this could be partly mediated by the proinflammatory effects of formula feeding in combination with microbial actions (19). In this study, formula feeding markedly decreased the proenzyme of caspase 1 (45kD, spot 3208, Fig. 4*A*; 20), a key marker of apoptosis, suggesting that the drive for apoptosis is less after feeding *in utero*. This could again be related to the lacking interaction of feeding with bacteria as the cytoskeleton rearrangement of enterocytes has been found to be related to bacterial colonization (21). Nevertheless, there is no doubt that feeding *in utero* induced significant changes in cellular structure-related proteins as well, as indicated by the differential expression of keratin 9 (spot 1202), 19 (spot 1302), and 10 (spot 3815) and actin $\gamma 2$ (spot 0210), $\alpha 2$ (spot 4305), and PRO 2619 (spot 4714).

Differentially regulated proteins present in our earlier studies (2,12), but not in this study, may represent proteins influenced by the birth transition, bacterial colonization, and early development of NEC. Among the proteins absent in this study are the stress proteins: heat shock protein (HSP) A5 and 27 (confirmed by Western blot, data not shown), which appeared in our previous studies with the existence of microbial colonization (2,12) showing that the differentiated expression of these proteins might depend on microbial colonization and/or inflammation. These changes are likely some of the early signs of formula-induced mucosal lesions, which, together with the action of bacterial stimuli and reperfusion injuries, may lead to NEC (22). In the current study, the trophic effect of formula *in utero* might be so mild that the fetal intestine did not have to recruit the HSPs to cope with the stress. Along the same lines, it is interesting that we found increased expression of guanine nucleotide binding protein (G protein), betapolypeptide 2-like 1 (GNB2L1, spot 8204) that may cause increased degradation of hypoxia-inducible factor-1α (HIF-1α; 23), whereas in postnatal preterm pigs formula feeding increased the HIF-1α expression (24). Moreover, proteins related to antioxidation, such as peroxiredoxin 5 (spot 6002) and glutathione synthetase (spot 3504) (25), were down-regulated after fetal formula feeding suggesting a limited drive for antioxidation and detoxification after fetal formula feeding. Finally, carbamoyl-phosphate synthetase 1 (spot 6902) that catalyzes the production of citrulline, arginine, and NO (26) were down-regulated in the intestine of formula-fed fetuses. The amino acids citrulline and arginine are known to play key roles for neonatal gut adaptation and mucosal homeostasis, partly because they are linked to NO synthesis, a pivotal molecule in maintaining tissue perfusion (27–29). Collectively, the above results indicate that fetal formula feeding does not up-regulate to the same extent as postnatal feeding up-regulate tissue markers of NEC such as HSPs and HIF-1α, as well as oxidative stress and tissue perfusion and homeostasis (arginine, citrulline, and NO).

In conclusion, the introduction of enteral formula feeding into the fetal intestine induced a remarkable growth and adaptation response, which in many aspects were similar to that induced by feeding of the preterm newborn after birth. The proteomic analysis of the intestinal proteins before and after fetal enteral feeding showed marked effects on pathways related to energy and nutrient metabolism, probably required to facilitate rapid changes in intestinal growth and function. However, we also identified a number of intestinal proteins that differed in response to feeding between the present fetal pigs and the earlier studies on preterm newborn pigs. Specifically in the fetal pigs, differential regulation of proteins related to bacterial colonization and NEC development was either absent or showed a direction of change not consistent with tissue inflammatory lesions.

REFERENCES

1. Bjornvad CR, Schmidt M, Peterson YM, Jensen SK, Offenberg H, Elnif J, Sangild PT 2005 Preterm birth makes the immature intestine sensitive to feeding-induced intestinal atrophy. Am J Physiol Regul Integr Comp Physiol 289:R1212–R1222
2. Jiang P, Sangild PT, Sit WH, Ngai HH, Xu R, Siggers JL, Wan JM 2009 Temporal proteomic analysis of intestine developing necrotizing enterocolitis following enteral formula feeding to preterm pigs. J Proteome Res 8:72–81
3. Jiang P, Siggers JL, Ngai HH, Sit WH, Sangild PT, Wan JM 2008 The small intestine proteome is changed in preterm pigs developing necrotizing enterocolitis in response to formula feeding. J Nutr 138:1895–1901
4. Siggers RH, Thymann T, Jensen BB, Molbak L, Heegaard PM, Schmidt M, Buddington RK, Sangild PT 2008 Elective cesarean delivery affects gut maturation and delays microbial colonization but does not increase necrotizing enterocolitis in preterm pigs. Am J Physiol Regul Integr Comp Physiol 294:R929–R938
5. Che L, Thymann T, Bering SB, LE Huërou-Luron I, D'inca R, Zhang K, Sangild PT 2010 IUGR does not predispose to necrotizing enterocolitis or compromise postnatal intestinal adaptation in preterm pigs. Pediatr Res 67:54–59
6. Sangild PT, Siggers RH, Schmidt M, Elnif J, Bjornvad CR, Thymann T, Grondahl ML, Hansen AK, Jensen SK, Boye M, Moelbak L, Buddington RK, Westrom BR, Holst JJ, Burrin DG 2006 Diet- and colonization-dependent intestinal dysfunction

predisposes to necrotizing enterocolitis in preterm pigs. Gastroenterology 130:1776–1792
7. Cilieborg MS, Boye M, Thymann T, Jensen BB, Sangild PT Diet-dependent effects of minimal enteral nutrition on intestinal function and necrotizing enterocolitis in preterm pigs. JPEN J Parenter Enteral Nutr, in press
8. Bjornvad CR, Thymann T, Deutz NE, Burrin DG, Jensen SK, Jensen BB, Molbak L, Boye M, Larsson L-I, Schmidt M, Michaelsen KF, Sangild PT 2008 Enteral feeding induces diet-dependent mucosal dysfunction, bacterial proliferation, and necrotizing enterocolitis in preterm pigs on parenteral nutrition. Am J Physiol Gastrointest Liver Physiol 295:G1092–G1103
9. Sangild PT, Trahair JF, Loftager MK, Fowden AL 1999 Intestinal macromolecule absorption in the fetal pig after infusion of colostrum in utero. Pediatr Res 45:595–602
10. Petersen YM, Burrin DG, Sangild PT 2001 Glp-2 has differential effects on small intestine growth and function in fetal and neonatal pigs. Am J Physiol Regul Integr Comp Physiol 281:R1986–R1993
11. Sangild PT, Mei J, Fowden AL, Xu RJ 2009 The prenatal porcine intestine has low transforming growth factor-beta ligand and receptor density and shows reduced trophic response to enteral diets. Am J Physiol Regul Integr Comp Physiol 296:R1053–R1062
12. Jiang P, Sangild PT, Siggers RH, Sit WH, Lee CL, Wan JM Bacterial colonization affects the intestinal proteome of preterm pigs susceptible to necrotizing enterocolitis. Neonatology, in press
13. Kumagai T, Nangaku M, Kojima I, Nagai R, Ingelfinger JR, Miyata T, Fujita T, Inagi R 2009 Glyoxalase I overexpression ameliorates renal ischemia-reperfusion injury in rats. Am J Physiol Renal Physiol 296:F912–F921
14. Ranganathan S, Walsh E, Godwin A, Tew K 1993 Cloning and characterization of human colon glyoxalase-I. J Biol Chem 268:5661–5667
15. Wright EM, Martin MG, Turk E 2003 Intestinal absorption in health and disease—sugars. Best Pract Res Clin Gastroenterol 17:943–956
16. Pearce DJ, Taussig D, Simpson C, Allen K, Rohatiner AZ, Lister TA, Bonnet D 2005 Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. Stem Cells 23:752–760
17. Satoh A, Nakajima Y, Miyahara I, Hirotsu K, Tanaka T, Nishina Y, Shiga K, Tamaoki H, Setoyama C, Miura R 2003 Structure of the transition state analog of medium-chain acyl-coa dehydrogenase. Crystallographic and molecular orbital studies on the charge-transfer complex of medium-chain acyl-coa dehydrogenase with 3-thiaoctanoyl-coa. J Biochem 134:297–304
18. Urata Y, Koike K, Goto S, Koike M 1991 Novel separation and amino acid sequences of alpha and beta subunits of pig heart pyruvate dehydrogenase. J Nutr Sci Vitaminol (Tokyo) 37:257–267
19. Oste M, Van Haver E, Thymann T, Sangild P, Weyns A, Van Ginneken CJ 2010 Formula induces intestinal apoptosis in preterm pigs within a few hours of feeding. JPEN J Parenter Enteral Nutr 34:271–279
20. Avivi-Green C, Polak-Charcon S, Madar Z, Schwartz B 2000 Dietary regulation and localization of apoptosis cascade proteins in the colonic crypt. J Cell Biochem 77:18–29
21. Petersen AM, Blom J, Andersen LP, Krogfelt KA 2000 Role of strain type, AGS cells and fetal calf serum in helicobacter pylori adhesion and invasion assays. FEMS Immunol Med Microbiol 29:59–67
22. Mallick IH, Yang W, Winslet MC, Seifalian AM 2004 Ischemia-reperfusion injury of the intestine and protective strategies against injury. Dig Dis Sci 49:1359–1377
23. Liu YV, Baek JH, Zhang H, Diez R, Cole RN, Semenza GL 2007 Rack1 competes with HSP90 for binding to hif-1α and is required for o2-independent and HSP90 inhibitor-induced degradation of hif-1α. Mol Cell 25:207–217
24. van Haver ER, Oste M, Thymann T, Sys SU, Lamers WH, Weyns AL, Sangild PT, van Ginneken CJ 2008 Enteral feeding reduces endothelial nitric oxide synthase in the caudal intestinal microvasculature of preterm piglets. Pediatr Res 63:137–142
25. Lu SC 2009 Regulation of glutathione synthesis. Mol Aspects Med 30:42–59
26. Dekaney CM, Wu G, Jaeger LA 2003 Gene expression and activity of enzymes in the arginine biosynthetic pathway in porcine fetal small intestine. Pediatr Res 53:274–280
27. Bailly-Botuha C, Colomb V, Thioulouse E, Berthe M-C, Garcette K, Dubern B, Goulet O, Couderc R, Girardet J-P 2009 Plasma citrulline concentration reflects enterocyte mass in children with short bowel syndrome. Pediatr Res 65:559–563
28. Oste M, Van Ginneken CJ, Van Haver ER, Bjornvad CR, Thymann T, Sangild PT 2005 The intestinal trophic response to enteral food is reduced in parenterally fed preterm pigs and is associated with more nitrergic neurons. J Nutr 135:2657–2663
29. Wu G, Jaeger LA, Bazer FW, Rhoads JM 2004 Arginine deficiency in preterm infants: biochemical mechanisms and nutritional implications. J Nutr Biochem 15:442–451